

# **PhD Theses**

## **Biophysical Characterisation and *in vitro* Digestibility of Sunflower Storage Proteins**

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## INTRODUCTION

The role of the plant seeds in human nutrition is diverse. Most important part of it is the consumption of seeds, partly the seeds of oil crops. Sunflower is the oil crop produced on the biggest area in Hungary, and also plays an outstanding role worldwide. Its seed contains oils and proteins in a great amount. After the oil extraction from the seeds there is a protein rich by-product left which can be used as forage, partly because of its high sulphur content.

Some storage proteins of sunflower seed are able to stabilise food emulsions, such as mayonnaise, due to their excellent emulsifying properties. Some of the storage proteins of sunflowers have been proved to elicit allergic reactions in sensitive individuals (Kean et al., 2006; Kelly and Hefle, 2002; Yagami, 2010), causing real danger to the patients. Due to the wide use of sunflower seeds establish a global risk to consumers.

A shared feature of allergenic food proteins is the ability to keep their structural integrity during food processing, consumption and digestion. The heat treatment and emulsification occurring during these processes may alter the some properties of the proteins and therefore their allergenicity. Better understanding of the gastro-intestinal processing, the digestion of protein emulsions is necessary for developing less allergenic food products.

Therefore we investigated those proteins of sunflower which had been found potentially able to stabilise food emulsions earlier (Burnett et al., 2002). We studied the lipid transfer protein of sunflower (LTP) and the SFA8 belonging to the 2S albumins, and three mixed fractions, comprised of mainly Alb1 and Alb2. We followed the change of the secondary structure upon heat treatment, the surface activity at the oil/water interface and their emulsifying properties. Both LTP (Yagami, 2010) and SFA8 (Kelly and Hefle, 2002; Kean et al., 2006) are able to elicit an allergic reaction in sensitive individuals. Alb1 and Alb2 possess such typical structure (the so called ‘LTP-signature’) which is representative to allergenic proteins, so presumably they might be allergenic as well. Therefore the allergenicity of the same protein fractions was also investigated: the proteins were digested in an *in vitro* gastro-intestinal model. The pattern and the timing of the proteolytic degradation of the proteins were investigated. We studied the effect of the emulsification and phosphatidylcholine, a lipid present *in vivo* in the gastro-intestinal tract, on the digestibility of the proteins.

In order to get better understanding if certain processes of food preparation affect the allergenicity of the studied proteins by modifying the resistance to proteolysis, we investigated how the structure of the proteins changes upon heat treatment.

The following questions were investigated:

- i) Can the proteins detailed above be used as emulsifiers? (What kind of surface activity they possess?)**
- ii) How does the surface activity change upon heat treatment imitating the food processing?**
- iii) Can these proteins cause allergy?**

In order to follow the alteration in the proteins' structure, to study their rheological properties and investigate their *in vitro* digestibility, we aimed the following points:

1. To study the changes occurring in secondary structure of the proteins.
2. To measure the surface activity of the proteins at the oil/water interface and study its changes upon heat treatment.
3. To characterise the emulsification properties of the proteins.
4. To study the resistance of the proteins against digestive enzymes (pepsin, trypsin, chymotrypsin) in an *in vitro* gastro-intestinal model.
5. To investigate the effect of a natural lipid on the digestibility of the proteins in the digestion model.
6. To study the effect the emulsification on the digestibility of the proteins.
7. To investigate the effect of the lipid-protein interactions (phosphatidylcholine and emulsification) on the digestibility and therefore the allergenicity.

## MATERIALS AND METHODS

Seeds of sunflower (*Helianthus annuus*, L., PR A381 hybrid line) were dehusked. 2S albumins and LTP were extracted by the method of Pandya et al. (2000).

This mixed so called ‘total 2S albumin fraction’ containing LTP as well was separated by reverse phase liquid chromatography (**RP-HPLC**). Five fractions were collected: **LTP**, **SFA8** and three mixed fractions: **fractions A, B and C**. The fractions contain mainly **Alb1** and **Alb2** proteins belonging to 2S albumins as well.

The purified proteins were studied by sodium-dodecyl-sulphate gelelectrophoresis (**SDS-PAGE**) (Shewry et al., 1995). The proteins were identified by **MALDI-TOF** technique and **N-terminal sequencing**.

### **Studies on molecular structure:**

We studied the change of the secondary structure of the proteins upon heat treatment by **Far-UV circular dichroism spectroscopy**. Spectra were recorded at 20°C then the samples were heated to 80°C for 20 mins and the spectra were recorded again. The samples were cooled back to 20°C for 20 mins in the spectropolarimeter *in situ* and new spectra were recorded. The analysis of the spectra was done by Contin method (Provencher and Glöckner, 1981).

We searched for the transition temperature of the proteins by **differential scanning calorimetry** (DSC).

### **Surface studies:**

Experiments were carried out on heat treated and native (control) samples as well. Measurements were performed at room temperature. All data points are averages of three parallel measurements carried out on independent drops.

The **surface tension** was measured at five different concentrations at hexadecane/water interface by pulsating drop method (Maldonado-Valderrama et al., 2008).

The **surface dilatation measurements** were carried out at air/water interface.

**Emulsions** were prepared by homogenisation of solutions of the five proteins and hexadecane. The size of the particles was measured. Three emulsions were made from all proteins and six independent measurements were carried out for all emulsions.

***In vitro* simulated gastro-intestinal digestion:**

In the 1<sup>st</sup> phase of the digestion the environment (composition, pH, temperature) of the stomach was modelled. The protein samples were digested in simulated gastric fluid by pepsin. After 2 hours of digestion the composition of the digesta was modified to the intestinal environment (2<sup>nd</sup> phase). The proteins were then digested by trypsin and chymotrypsin for 2 more hours. The digestion was carried out in the presence and absence of phosphatidylcholine vesicles (liposomes). Samples were taken from the digesta 10 times and separated on SDS-PAGE.

## RESULTS AND DISCUSSION

1. We have found that only small changes occurred in the molecular structure of the 2S albumins of sunflower on heat treatment. The structure of these proteins remained stable. The structure of LTP was stable as well. It showed only minor changes upon heat treatment but after heating it did not refold to its original conformation perfectly.

2. The surface activity measured on the oil/water interface was affected by the applied heat treatment of the five studied fractions of sunflower storage proteins. The heat treatment increased the surface activity largely in case of LTP. In case of the mixed fractions (A, B and C, which contain Alb1 and Alb2 proteins) and the SFA8 the surface activity was raised in a smaller extent by heat treatment. Only the SFA8 exhibited excellent surface activity, the LTP and the three mixed fractions (A, B and C) showed poorer surface properties.

3. LTP was able to stabilise emulsions to a modest extent, while the mixed fractions were not able at all. Only the SFA8 was able to form stable emulsions, its emulsions possessed outstanding properties, such as small drop size, long term stability.

4. We studied the resistance of the proteins against proteolysis in an *in vitro* digestion model. Two phases of the physiological digestion were simulated: the gastric phase occurring in the stomach (1<sup>st</sup> phase) and the intestinal phase which takes place in the small intestine (2<sup>nd</sup> phase). We found during the *in vitro* digestion:

4.1. that all the proteins of sunflower we investigated in this study (LTP as well as the 2S albumins) are digested in the artificial gastro-intestinal model;

4.2. LTP and the 2S albumins retain their structural integrity even after being cleaved by the proteases because the fragments are held together by disulphide bridges during the gastro-intestinal digestion. Presumably, the fragments are able to get to the small intestine and pass through its mucosa eliciting and allergic reaction.

5. Phosphatidylcholine had a defensive effect both in gastric and intestinal phase of digestion on LTP as well as the 2S albumins of sunflower: the mixed Alb1 and Alb2 fractions and pure SFA8.

6. Emulsification provided an increased protection for SFA8 against proteolysis. (Only SFA8 is able to stabilise emulsion to sufficient extent amongst the proteins studied here.)

7. The protection was most efficient when the protein (SFA8) was emulsified and phosphatidylcholine was added to the digesta.

It indicates that both the **lipid-protein interactions and the emulsification can modify a protein's allergenicity.**

## CONCLUSIONS

As a conclusion, it is necessary to take into consideration when assessing novel proteins

1. besides of the pepsin digestion the proteolysis of trypsin and chymotrypsin as well;
2. the multiphase nature of the gastro-intestinal tract
3. and the lipid-protein interactions.

**i-ii) The four studied proteins of sunflower, especially SFA8, possess good surface properties which are further enhanced by heat treatment, therefore SFA8 could be used as emulsifier.**

**iii) Due to the proteins' allergenicity their use as stabilisers in food emulsions should be thoroughly considered.**



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## LIST OF PUBLICATIONS THE THESES ARE BASES ON

### A. Papers published in peer-reviewed journals

1. **Berecz, B.**, Mills, E.N.C., Parádi, I., Láng, F., Tamás, L., Shewry, P.R., Mackie, A.R. (2013) Stability of Sunflower 2S Albumins and LTP to Physiologically Relevant *in vitro* Gastrointestinal Digestion. *Food Chemistry*, 138: 2374-2381. [dx.doi.org/10.1016/j.foodchem.2012.12.034](https://doi.org/10.1016/j.foodchem.2012.12.034) **IF: 3,655**
2. **Berecz, B.**, Mills, E.N.C., Tamás, L., Láng, F., Shewry, P.R., Mackie, A.R. (2010) Structural Stability and Surface Activity of Sunflower 2S Albumins and Nonspecific Lipid Transfer Protein. *J. Agric. Food Chem.* 58: 6490–6497. [dx.doi.org/10.1021/jf100554d](https://doi.org/10.1021/jf100554d) **IF: 2,816**
3. **Berecz, B.**, Mackie, A.R., Shewry, P.R., Tamas, L., Mills, C.E. (2005) Interfacial properties of sunflower storage proteins. *FEBS Journal* 272: 366-366. **IF: 3,164**

### B. Publications in conference proceedings

1. Parádi, I., Bratek, Z., **Berecz, B.**, Halász, K. (2002) Influence of arbuscular mycorrhiza, P limitation and Cd-stress on polyamine contents of plants. Proceedings of the 7<sup>th</sup> Hungarian Congress on Plant Physiology. *Acta Biologica Szegediensis* 46: 59-60.
2. **Berecz, B.**, Bratek, Z., Halász, K., Rudnóy, Sz., Parádi, I. (2000) Mycorrhizal fungi of the bauxite mine spoil at Gant, Hungary. *Acta Biologica Debrecina Oecologica Hungarica* suppl. 11(1): 197.

### C. Other peer-reviewed publications not connected to the topic of the theses

1. Parádi, I., Bratek, Z., **Berecz, B.**, Halász, K., Láng, F. (2003) Effect of Cd stress and mycorrhiza on polyamine content of Ri T-DNA transformed carrot root cultures. *Acta Biologica Szegediensis*, 47: 31-36.